PEPTIDE INHIBITING PLATELET DERIVED GROWTH FACTOR (PDGF-BB) AND FIBROBLAST GROWTH FACTOR (bFGF) ACTIVITY.

The present invention concerns the identification and the synthesis of a peptide, derived from the basic human fibroblast growth factor (bFGF), having the following primary structure:

Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu

hereafter referred to as PEP1.

Said molecule, showing analogy with a sequence of bFGF, namely inhibits *in vitro* as well as *in vivo* PDGF-BB and bFGF effects.

More particularly, *in vitro* experimentation on primary rat smooth muscle cells (RASMC) and primary bovine endothelial cells (BAEC) indicated that said molecule is an efficient inhibitor of cell proliferation and migration at a dose that is not toxic for cells.

Moreover, *in vivo* experimentation carried out on reconstituted basement membrane plugs, subcutaneously injected in CD1 mice demonstrated that said molecule strongly inhibits bFGF-induced angiogenesis.

Reported results suggest that PEP1 might be used for the treatment of diseases with abnormal proliferation and migration of vascular cells such as restenosis after angioplasty, atherosclerosis, tumor growth and metastasis dissemination.

Growth factors, such as Platelet Derived Growth Factor (PDGF-BB) and basic Fibroblast Growth Factor

(bFGF) play a crucial role in the proliferation and differentiation of many cell types. In fact, increased levels and/or activity of these factors occur in several pathologies, including tumor growth and blood-vessel diseases like atherosclerosis.

Platelet Derived Growth Factor (PDGF-BB) and basic Fibroblast Growth Factor (bFGF) are both essential for the phatogenesis of angiogenesis-related diseases since they directly modulate cell proliferation and migration within vascular wall (Ross, R., et al. 1990, Science, 248, 1009-1012; Ross, R. 1993, Nature, 362, 801-809).

Angiogenesis is a key process for tissue development, as well as tumor growth and dissemination. It is controlled by several factors modulating cell differentiation, proliferation and migration (Holash, J., 1999, Oncogene, 18, 5356-5362; Zetter, B.R. et al., 1998, Annu. Rev. Med., 49, 407-424).

Several different molecules, such as antibodies neutralising PDGF and bFGF (Rutherford et al., Atherosclerosis, 1997, 45-51) and oligonucleotides inhibiting PDGF receptor expression (Sirois, M.G. et al., 1997, Circulation, 95, 669-676), were successfully used *in vivo* to inhibit diseases with abnormal proliferation and migration of vascular cells such as restenosis. Furthermore, specific inhibitors currently available are able to interfere with the receptor-binding or receptor dimerization or signaling (Heldin, C.H. et al., 1998, BBA, F79-F113).

PDGF and bFGF are required for tumor cells growth *in vitro*, growth of solid tumors *in vivo*, as well as

metastases dissemination (Shawver, L.K. et al., 1997, Clin. Cancer Res., 3, 1167-1177; Vignaud, J.M. et al., 1994, Cancer Res., 54, 5455-5463; Chandler, L.A. et al., 1999, Int. J. Cancer, 81, 451-458; Westphal, J. R. et al., 2000, Int. J. Cancer, 15,86 (6), 768-776).

Inhibiting the activity and/or the signaling of PDGF and bFGF led to effective reduction of tumor growth and metastasis dissemination (Abramovich, R. et al., 1999, Br. J. Cancer, 79 (9-10), 1392-8; Bagheri-Yarmand, R. et al.,1998, Br. J. Cancer, 78 (1), 1118; Sola, F. et al, 1995, Invasion Metastasis, 15 (5-6), 222-231; Wang, Y. et al., 1997, Nature Med., 3, 887-893).

Therefore, specific antagonists of PDGF and bFGF are potential candidates for the treatment of proliferative diseases and angiogenesis-related disorders.

According to recent data collected by the same inventors, PDGF-BB and bFGF play an unsuspected role in the modulation of their pro-angiogenic functions. In particular, the inhibitory role of bFGF on cell proliferation and migration in addition to its pro-angiogenic effect, has been demonstrated (Facchiano, A. et al., 2000, J. Cell. Sci., 113, 2855-2863).

Moreover, the factors regulating the protein-folding and the structure-biological function relationship has been investigated (Ragone, R. et al., 1987, Italian J. of Biochem., 36, 306-309; Facchiano, F. et al., 1988, CABIOS, 4, 2, 303-305; Ragone, R. et al., 1989, Protein Engineering, 2, 7, 497-504;

Facchiano, A. M. et al., 1989, CABIOS, 5, 4, 299-303; Facchiano, A.M. et al., 1991, CABIOS, 7, 3, 395- 396; Facchiano, A. et al., 1993, J. Mol. Evol., 36 (5), 448-457; Benvenga, S. et al., 1993, EOS-J. of Immunol. and Immunopharm., 13 (1), 18-19; Facchiano, A., 1995, J. Mol. Evol., 40, 570-577; Facchiano, A., 1996, Trends in Genetics, 12(5), 168-169; Scarselli, M. et al., 1997, J. Peptide Sci., 3, 1-9; Benvenga, S. et al., 1999, Amyloid, 6 (4), 250-255; Facchiano, A.M., 1999, Protein Eng., 12 (10),893; Pozzetto, U. et al., 2000, Transplant Int., Suppl. n. 1, 13, S306-S310; Facchiano, A. M., 2000, Bioinformatics, 16 (3), 292-293).

In the present invention, by investigating protein structure, regions of bFGF sequence potentially responsible of its biological activity have been identified. Among these regions, a peptide having the following primary structure:

Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu

(here referred to as PEP1), derived from human bFGF turned out to be a strong inhibitor *in vitro* of bFGF, PDGF-BB and fetal calf serum (FCS) effects, such as cell proliferation and migration observed in primary rat smooth muscle cells (RASMC) and primary bovine endothelial cells (BAEC). Said activity has been observed at a dose as low as 10 nanograms/milliliter and PEP1 is not toxic at this dose *in vitro*. The heat-denatured and the scrambled version (with random aminoacid sequence) of PEP1 were used as control: both do not show any activity.

Moreover, PEP1 even show inhibitory activity *in vivo*; it is, indeed, able to inhibit angiogenesis in reconstituted basement membrane plugs, subcutaneously injected in CD1 mice.

Accordingly with what previously detected, PEP1 synthesis was achieved by automatic synthetizer, using the standard technique named f-moc.

After that, three different batches of PEP1 were tested and they gave similar results in the biological assays. Moreover, a scrambled version of the peptide (PEP1scr) was prepared and after used as negative control in all the experiments.

Several *in vitro* and *in vivo* test were carried out on said molecule and they revealed the functional characteristics of said peptide.

The results obtained are reported in the accompanying drawings:

Figure 1 shows the results of dose-dependent experiments carried out on RASMC. RASMC proliferation induced by 10% FCS was evaluated after 48 hours, in the absence and in the presence of different PEP1 doses, ranging from 1g/mi to 1 pg/ml;

Figure 2A shows PEP1 and PEPscr effect on RASMC proliferation induced by PDGF-BB (10ng/ml);

Figure 2B shows PEP1 and PEP1scr effect on RASMC spontaneous proliferation in the presence of BSA;

Figure 3A shows PEP1 and PEPscr effect on BAEC proliferation induced by PDGF-BB (10ng/ml);

Figure 3B shows PEP1 and PEP1scr effect on BAEC spontaneous proliferation in the presence of BSA;

Figure 4A shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by FCS (1%);

Figure 4B shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by PDGFD-BB (10 ng/ml);

Figure 4C shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by bFGF (10 ng/ml);

Figure 5A shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by EGF (10ng/ml);

Figure 5B shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by aFGF (10ng/ml);

Figure 5C shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by Fibronectin (10ng/ml);

Figure 5D shows the effect in the presence or in the absence of PEP1 and PEPscr (10ng/ml) on BAEC migration induced by VEGF (10ng/ml);

Figure 6 shows PEP1 and PEP1scr effect on RASMC migration induced by PDGF-BB (10ng/ml);

Figure 7 shows PEP1 and PEP1scr effect on angiogenesis induced by bFGF in reconstituted basement membrane plugs, subcutaneusly injected in CD1 mice.

IN VITRO PEP1 ACTIVITY ASSAY

This test was carried out on Primary rat aorta smooth muscle cells (RASMC) obtained from six-month old male Wistar rats following a well known technique

(Sterpetti, A. V. et al., 1992, J. Vasc. Surg., 6, 16-20); primary bovine aortic endothelial cells (BAEC) were obtained according to previously described protocols (D'Arcangelo, D. et al., 2000, *Circ.Res.*,86,312-318).

MIGRATION ASSAY

Cell migration is a key process for the development of new blood-vessels. Consequently, PEP1 effect on cell migration induced by several different chemoattractant factors has been evaluated mainly on endothelial cells (BAEC). Migration assays were carried out in modified Boyden chambers (Neuroprobe Inc.), following known standard techniques (Albini, A. et al., 1995, Int. J. Cancer,61,121-129; Facchiano, A. et al., 2000, J. Cell. Sci., 113, 2855-2863). Cells were dispensed in the upper portion of the Boyden chamber. Chemoattractant factor were calf fetal serum (FCS) 10% or the following human recombinant factors: PDGF-BB, bFGF and vascular endothelial growth factor (VEGF). PEP1 PEPscr (scrambled control) diluted in water, were added to the growth factor solution at the reported final concentration. Thus chemotaxis induced by bFGF (10ng/ml), or PDGF-BB (10ng/ml), or FCS (2%), in the absence or in the presence of 10ng/ml PEP1 and PEP1scr, was evaluated.

All the migration assays were carried out at 37°C in 5% CO_2, for a total time of 5 hours; then filters were removed, fixed with absolute ethanol and stained with toluidine blue. Cells migrated were counted at 400X magnification in 15 fields for each filter and the

average number of cell/field was reported. All the experiments were performed at least 3 times in duplicate.

The experiments show that, in every condition, PEP1 markedly inhibit, and in a rate more than 50%, BAEC migration, but PEP1scr do not have any effect (Figure 4A, 4B e 4C). When bFGF or PDGF-BB were tested, PEP1 was either dispensed in the lower and in the upper portion of the Boyden chamber; a slightly better inhibitory activity was observed when it was dispensed in the lower portion of the Boyden chamber.

In contrast, PEP1scr control does not show any activity when dispensed in both portion of the Boyden chamber. To evaluate the specificity of said inhibitory effect, PEP1 effect on other chemoattractans was tested. PEP1 and PEP1scr do not affect Endothelial cell migration induced by aFGF or VEGF or EGF or Fibronectin (Figures 5A, 5B, 5C and 5D), indicating that said molecule specifically affect bFGF and PDGF-BB.

Similar results were obtained in chemotaxis assays carried on RASMC induced by PDGF-BB and FCS. PEP1 inhibits RASMC migration (1.e. about 60%), while PEP1scr is inactive (Figure 6).

PROLIFERATION ASSAY

Proliferation assay was carried out on primary rat aorta SMC and on primary bovine aortic endothelial cells (BAEC). Cells were plated in six-well plates ($1x10^5$ cells/plate) and grown for 24 hours in Dulbecco Modified eagle's medium (DMEM) supplemented with 10% FBS, at 37°C in 5% CO_2. Then, the medium was replaced

with DMEM medium containing 0.1 % BSA for 24 hours. Subsequently, the medium was replaced with fresh medium containing either 0.1 % BSA alone or 0.1 % BSA with growth factors at 10 ng/ml final concentration or fetal calf serum (FCS) al 10%, in the absence or in the presence of PEP1 or control peptide. Each assay was carried out for mounting period of time up to a maximum time of three days and the cell were harvested and counted with hemacytometer.

First of all, PEP1 was tested in dose-dependence experiments: RASMC proliferation induced by FCS 10%, was evaluated at 48 hours, in the presence and in the absence of different PEP1 doses, ranging from 1 µg/ml to 1 pg/ml (figure 1). The heat-denatured PEP1 and the scrambled version of PEP1 were used as control. PEP1 showed a dose-dependent inhibitory activity, which reached 60% inhibitory effect at 10ng/ml, while the control peptides did not show any activity. Consequently, the dose of 10ng/ml was chosen for the following *in vitro* experiments.

The effect of PEP1 was tested on proliferation induced by PDGF-BB and bFGF (10ng/ml each), in RASMC and BAEC. Figure 2A shows the marked inhibition of proliferation induced by PDGF-BB. In time course experiments, proliferation induced by PDGF-BB (10ng/ml) was significantly inhibited in the presence of PEP1 at all time points. PEP1 block almost completely cell proliferation, while the control scrambled peptide (PEP1scr) is not effective at any time (figure 2A).

Spontaneous proliferation (in the presence of bovine serum albumin, BSA) is not significantly affected by PEP1 nor by PEP1scr at any time, indicating that both molecules are not toxic per se at the tested doses on RASMC (Figure 2B), nor on BAEC (figure 3B). Moreover, PEP1 shows similar inhibitory effect on BAEC stimulated by bFGF (10ng/ml) (Figure 3A).

Then the following *in vivo* experiment was carried out:

ANGIOGENESIS ON RECONSTITUTED BASEMENT MEMBRANE PLUGS

Angiogenesis on reconstituted basement membrane plugs (named "Matrigel", produced by Collaborative Biomedical Products, Beckton-Dickinson) was carried out as previously reported (Muhlhauser, J., 1995, *J. Circ. Res.*, 77, 1077-1086). Briefly, reconstituted basement membrane plugs added with bFGF (150 ng/ml) alone or in the presence of PEP1 (10 micrograms/ml) were subcutaneusly injected in CD1 mice (female, 19 weeks age). bFGF induces the formation of capillary network within 7 days, therefore plugs were excised 7 days after injection and included in paraffin. Obtained slides were stained with trichrome-Masson staining procedure and analysed with an optical image analizer and the number of vessels per mm^2 within plugs was quantified.

Figure 7 shows that PEP1 acts as strong inhibitor of blood vessel formation induced by bFGF (i.e. 46% inhibition vs bFGF alone). 10 animals were used as control (treated with bFGF alone) and 14 animals were

treated with bFGF in the presence of PEP1. This experiment shows that PEP1 is able to markedly inhibit new-blood vessel formation induced by bFGF and indicates PEP1 as a good candidate for further *in vivo* studies.

In conclusion:

1) PEP1 showed a strong and specific inhibitory activity on mitogenic and chemoattractive properties of platelet derived growth factor (PDGF-BB) and fibroblast growth factor (bFGF) *in vitro*.

2) Anti-angiogenic activity *in vivo* was demonstrated in assays carried out on reconstituted basement membrane plugs.

These results indicate PEP1 as a good candidate for further investigation on animal models of tumor growth and metastasis as well as other vascular-based diseases.